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13. ABSTRACT *(Maximum 200*
Tyrosine kinases mediate cellular responses to environmental conditions, and they are involved in regulation of proliferation and cell differentiation in a variety of systems. Overexpression or mutation of tyrosine kinases has been shown to result in cellular transformation, and this has been seen in breast tumors. Our laboratory identified a novel mouse epithelial cell specific intracellular tyrosine kinase, which we named Sik, for **S**rc-related **i**ntestinal **k**inase. We isolated the human homologue of Sik and determined that it is identical to a novel tyrosine kinase termed Brk (**Br**east tumor **k**inase). Brk was isolated from a metastatic breast tumor, and its expression had only been detected in human breast tumors and breast tumor cell lines. We have detected Brk expression in the normal human small and large intestine, and are examining its expression during normal breast development. We have determined that expression of Sik/Brk is first induced as epithelial cells stop dividing and terminally differentiate, and we hypothesize it may regulate an aspect of normal epithelial cell differentiation. We are trying to determine if targeted expression of normal or mutant forms of Sik/Brk in mammary epithelial cells can influence growth or differentiation. We have found that Brk mRNA and protein are expressed at higher levels in some breast tumors, than in normal tissue. We are now examining Brk expression and activity in different types of breast tumors to determine if Brk expression may provide a diagnostic or prognostic marker for the development of breast cancer.

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Angela L Lynn 9/18/97

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INTRODUCTION

Tyrosine kinases include transmembrane receptors for many growth factors and intracellular kinases that transmit signals from the membrane to the nucleus (1). They are involved in the regulation of proliferation and cell differentiation in a variety of systems (2, 3). Aberrant expression of tyrosine kinases is linked to oncogenesis. In addition, several classical developmental mutations have been mapped to tyrosine kinase genes, underscoring the important role that these proteins play during differentiation (for reviews see 4-7).

To gain insight about the signal transduction pathways regulating the rapid turnover and constant differentiation of the intestinal epithelium, we previously cloned portions of the catalytic domains of tyrosine kinases expressed in the mouse small intestine, using the polymerase chain reaction with degenerate primers and intestinal cDNA (8). We identified a novel tyrosine kinase with homology to Src in our screen, and we named it Sik, for **S**rc-related **i**ntestinal **k**inase (8). A full length cDNA encoding mouse Sik was cloned and characterized (9). Like Src, Sik contains SH3 and SH2 domains, and a putative regulatory tyrosine at the carboxy terminus (9). In contrast to the Src family members, Sik has a very short unique domain at the amino terminus and it is not myristoylated. Unlike Src, which is expressed in the crypts of the small intestine, expression of Sik is initiated as cells migrate from the crypts and terminally differentiate. The tissue distribution of Sik is much more restricted than that of Src and the other Src family members expressed in the intestine.

Expression of Sik is developmentally regulated (9), and first appears in the mouse at day 15.5 of gestation, in the granular layer of the skin. We detected striking expression of Sik in the outer layers of the mouse forestomach at 17.5 days of gestation. Sik expression rises in the intestine as cell proliferation becomes restricted to the crypts near the time of birth. Expression of Sik always appears to be initiated in epithelial cells of the skin and gastrointestinal tract as they stop dividing and start terminally differentiating (9). This pattern of expression suggests that Sik may be required for normal differentiation of regenerating epithelial cell linings.

To isolate the human homologue of Sik, we used the mouse Sik cDNA clone to screen a normal human small intestine cDNA library that was purchased from Clontech. From a screen of 10^6 recombinant phage, three positive clones were isolated. When sequenced, these partial cDNA clones shared sequence identity with a novel nonreceptor tyrosine kinase, termed Brk (breast tumor kinase) that was isolated from a human metastatic breast tumor (10). The Brk tyrosine kinase was initially identified using PCR and degenerate primers corresponding to the conserved

regions of tyrosine kinase catalytic domains, and RNA isolated from involved axillary nodes from a patient with metastatic breast cancer (10). The cloned Brk catalytic domain was used to screen a cDNA library prepared from the T-47D breast cancer cell line and Brk cDNA clones were isolated. Expression of Brk was examined in normal breast from reduction mammoplasty and in stage III breast tumors (grading from the Dept. of Health & Royal College of Pathologists Working Group 1989). In the initial study, Brk was detected only in the tumor tissue and in the T-47D and MCF-7 human breast cancer cell lines, but not in normal breast (10). Recently it has been reported that Brk is expressed in a high proportion of human breast carcinomas (11).

Like Sik, Brk is also a 451 amino acid protein with common sequences found in Src family kinases, such as SH2 and SH3 domains and a putative regulatory tyrosine at the carboxy terminus. Sik/Brk share significant homology with Rak/Frk (44% identity) another new epithelial cell kinase also expressed in breast tumor cells (12, 13). In contrast to members of the Src family, Sik, Brk, and Frk do not contain sites for myristoylation at the amino terminus. Sik, Brk and Frk also have the sequence HRDLAARN in their catalytic domains, in contrast to the sequence HRDLRAAN shared by members of the Src family.

While the functional domains of Brk and Sik are conserved, Sik and Brk share only 80% sequence identity. Because of the relatively low level of sequence identity between Sik and Brk, we performed a series of Southern hybridization experiments to confirm that Sik and Brk are true homologues of one another. ^{32}P-labeled *brk* and *sik* probes were hybridized with restricted mouse and human genomic DNAs. If a gene more closely related to *sik* than *brk* was present in the human genome, bands corresponding to both of these genes would hybridize with the *sik* probe. Similarly, if the mouse genome contained another gene more closely related to *brk* than *sik*, additional more strongly hybridizing bands corresponding to this gene would appear after hybridization of mouse genomic DNA with the *brk* probe. Both the mouse *sik* probe and the human *brk* probe detect the same fragments in mouse and human genomic DNA, confirming that Sik and Brk are homologues of one another.

We hypothesize that brk (sik) is involved in signal transduction breast epithelial cells, and that its aberrant expression may be important in the development of breast tumors. In the work proposed, we will focus on gaining a better understanding of the role of this newly identified epithelial cell specific tyrosine kinase. We have generated a variety of Sik expression constructs which will be used to perform functional studies to learn about the mechanism of brk/sik action in vitro and in vivo. By determining the precise patterns of brk expression in the developing mammary gland, and in human

breast tumors, we may find that brk will provide a diagnostic or prognostic marker for the development of breast cancer.

BODY

Generation of wildtype and mutant Sik expression constructs

To determine the biological activity of the Brk(Sik) kinase, three constructs have been prepared which will be expressed in mammary epithelial and NIH 3T3 cell lines, including: 1) wildtype brk; 2) a dominant negative form of brk in which the catalytic domain has been mutated, and 3) a constitutively activated form of brk in which the regulatory carboxy tyrosine has been altered. We will determine if Brk(Sik) expression in cell lines results in altered growth characteristics, cellular transformation or induction of differentiation.

We have successfully generated the wildtype and mutant Sik expression constructs to be used for examining Sik function in various cell lines. The full length *sik* cDNA cloned into the pBluescript II SK vector was used in the construction (9). First, the *sik* cDNA was subcloned into the pAlter vector (Promega, Madison, WI) using BamHI and KpnI sites. Then, the 3'-nontranslated region was deleted by partial digestion with SstI and self-ligation. Next, the *sik* coding sequence was cloned into the EcoRI site of the pLXSN retroviral expression vector (14).

The oligonucleotide-mediated Altered Sites in vitro Mutagenesis System (Promega Corp., Madison, WI) was used for preparation of the mutant kinase deficient sik cDNA. The complete sik cDNA was cloned into the pAlter vector using BamHI and SalI sites. The oligonucleotide used for catalytic domain mutagenesis 5'-GTGGCTGTGATGGTGATCTCT-3' has a substitution of A for T which changes the lysine at position 219 of wild type Sik to methionine. A second oligo, 5'-CACCAGGTTTGAGAACC-3', which has a substitution of A for T which changes the tyrosine at position 447 to phenylalanine was used for mutation of the putative regulatory carboxy tyrosine. The entire sequence of the mutant cDNAs was confirmed by sequencing with Sequenase (USB, Cleveland, OH) using dideoxynucleotide chain termination method (15).

Transfection of the normal murine mammary gland cell line NMuMG has been done as previously described (16). Briefly, the BOSC 23 packaging cell line was transiently transfected with plasmids using the $CaPO_4$-precipitate procedure. Within 48 hours after transfection the supernatant from these cells was used for infection of the NMuMG cell line. In experiments with control plasmid MFG-lacZ, encoding β-galactosidase (17), between 30 and 60% of the cells were infected. At 48 hours after

infection the cells were split 1:4 into selective media containing 250 μg/ml of active G418 (Gibco BRL) and were refed every 2 days. Upon reaching the confluence the cells were split 1:4 once again. After 2 weeks in selective media there were no dead or dying cells in the culture.

We are currently using the resulting cell lines in experiments as a pools of stably transfected clones. We are in the process of examining the growth properties of these cells. In preliminary experiments, we have not detected obvious phenotypic changes. We have examined the localization of overexpressed Sik in these cells and in primary mouse keratinocytes and this is described below.

Generation of Sik GST fusion proteins

Sik/Brk is composed of SH1 (catalytic), SH2 (phosphotyrosine-binding), and SH3 (poly-proline) binding domains. To facilitate identification of interacting proteins, we have generated pGEX-Sik expression constructs and GST fusion proteins containing each of the different domains of Sik. An expression construct containing the full length and the 3'-nontranslated terminus of the sik cDNA was generated by cloning a NcoI/XhoI fragment into corresponding sites of the pGEX-KG expression vector (18). The construct for expression of the GST-Sik SH3 domain was made by PCR amplification using oligonucleotides 5'-CAAGCAGGCCACAGCTGACT-3' and 5'-GCGCTCGAGTCACACAGTTTCCTTCTCAGCCA-3' and the sik cDNA as template. The amplified fragment was digested by NcoI and XhoI and subcloned into pGEX-KG. For expression of the GST-Sik SH3/2 domain, the NcoI/Eco47III fragment of the *sik* cDNA was subcloned first into pet25b(+) vector (Novagen, Madison, WI) using NcoI and a filled-in Not I site. The NcoI and XhoI fragment from this plasmid was then subcloned into pGEX-KG. The construct for expression of the GST-Sik SH2 domain was made by PCR amplification using oligonucleotides 5'-GCGCCATGGAACCGTGGTTCTTTGGTT-3' and 5'-GCTAGTTATTGCTCAGCGG-3' and the previously described pet25b(+) plasmid containing SH3/2 domains of sik as a template. The amplified fragment was digested with NcoI and XhoI and ligated into the corresponding sites of pGEX-KG. All PCR amplifications are done using Taq polymerase (Promega, Madison, WI) as described elsewhere (19). The integrity of all expression constructs was verified by sequencing.

The fusion proteins are expressed in the DH5α strain of *E. coli* essentially as described (18). Protein expression is induced by addition of IPTG up to 0.1 mM at 20°C (at 10°C for GST-Sik) for 2 hours Cells are disrupted by sonication and fusion protein is affinity purified using glutathione-sepharose (Pharmacia Biotech Inc.,

Piscataway, NJ). The bound proteins are eluted by 20 mM reduced glutathione, dialyzed against PBS containing 10% glycerol, 5 mM DTT and stored at -70°C. A GST-GRB2 fusion protein control was purchased from Santa Cruz Biotechnology.

Using extracts from breast and other epithelial cell lines, we are currently trying to identify proteins that bind to Brk(Sik). We are trying to determine the identity of proteins that co-immunoprecipitate with Sik and that bind to the different GST-fusion proteins.

Brk(Sik) expression in mouse mammary epithelial cells

To determine the patterns of *brk* expression during normal mammary gland development in the mouse, we have been using the mouse *brk(sik)* cDNA as a probe in in situ hybridization experiments. We have isolated mammary gland tissue from virgin, pregnant, and lactating, and mature females after involution for these studies. In situ hybridizations have been performed as described in Serfas and Tyner, 1993 (20), using both frozen and paraffin embedded tissues. In situ hybridization provides a sensitive means for detecting expression in a small number of cells within a tissue. Thus far, our results indicate that *brk(sik)* is expressed at either a very low or undetectable level in most normal breast tissue. We have obtained a hybridization signal over epithelium only in breast tissue from lactating females. These studies are currently being repeated. Comparison of these results with results from in situ hybridization with intestine and skin indicate that *brk(sik)* is expressed at a significantly lower level in the breast epithelium.

To examine Brk(Sik) protein expression and activity we prepared anti-Sik antibodies using recombinant Sik fusion protein representing the junction region between the Sik SH2 domain and catalytic domain, and a peptide corresponding to the 18 C-terminal amino acids of Sik (CFKDLCEKLTGITRYENL) as immunogens. The polyclonal antisera prepared against the Sik fusion protein inhibited Sik kinase activity, while the antibody generated against the peptide did not work for immunoprecipitations. Subsequently, anti-Sik antibodies sc-915 and sc-916, directed against different parts of the Sik protein became available (Santa Cruz Biotechnology). We tested these antibodies and found that the sc-916 antibody was able to immunoprecipitate in vitro translated Sik and did not interfere with Sik kinase activity. Specificity of sc-916 was verified by immunoprecipitation using protein extracts cells expressing Sik from the pLXSN vector. The corresponding 53 kDa Sik protein was immunoprecipitated only from cells transfected with Sik construct and not vector alone. The sc-916 antibody will be used for all Sik immunoprecipitation experiments. The sc-

915 antibody does not work for immunoprecipitation of Sik, but worked for immunoblotting. Western blot analyses and immunohistochemistry are performed with a combination of the two Santa Cruz Biotechnology antibodies, sc-916 and sc-915, for increased sensitivity.

Using immunohistochemistry and western blotting, we have localized the Sik protein in a variety of cell lines, and we have determined that Sik is primarily found in the nucleus in primary keratinocyte cultures. In cell lines expressing transfected Sik, including the murine mammary gland cell line NMuMG and in HT-29 cells, Sik protein can be found throughout the cell, including the nucleus. Interestingly, Sik protein in the NMuMG cells appears in distinct foci. We are now trying to determine the identify of the foci. The appearance of Sik in the nucleus suggests that Sik may have specific nuclear substrates.

Characterization of the Brk polyclonal antibody and examination of Brk expression patterns in human tissues

In order to generate an antibody that would recognize Brk, we ordered a Brk peptide corresponding to 17 carboxy terminal amino acids of the Brk protein and this peptide was injected into rabbits to produce a polyclonal anti-sera. The peptide and antisera were prepared by Research Genetics, Huntsville, Alabama. We affinity purified Brk anti-bodies from the sera received from Research Genetics. In control western blots the Brk polyclonal anti-sera was not highly specific or reactive. As we were testing the polyclonal Brk antisera that we generated, an anti-Brk antibody also became commercially available from Santa Cruz Biotechnology, Inc. The commercially available antibody had much greater specificity than the antibody that we prepared and were in the process of characterizing. For expression studies, we have been using the commercially available Santa Cruz Brk antibody (sc-1188).

To examine Brk expression in breast tumors, we have been using indirect immunohistochemistry with tyramide amplification. Tyramide amplification greatly amplifies an antibody signal (21), enabling us to detect low levels of Brk protein expression. Paraffin-embedded biopsy samples sectioned at 5-8 micrometers thick are deparaffinized, hydrated, and preincubated in block buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% TWEEN 20, 1:50 normal goat serum) for 40 minutes. Sections are then incubated with 0.2 ug/ml Brk antibody (Santa Cruz) in block buffer overnight at 4 C, washed, and incubated with 1:250 biotinylated goat anti-rabbit antibody (Vector) in block buffer for 30-60 min. After washing, the TSA-indirect kit (DuPont/NEN) is used according to manufacturer's instructions. Briefly, sections are

treated with strepavidin-horseradish peroxidase, reacted for 5 minutes with biotinyl tyramide reagent, visualized with 1:500 FITC-Avidin DCS (Vector), and mounted with Vectashield mounting medium (Vector). For control sections, 4 ng per ng of Brk antibody, of the peptide from which the Brk antibody was raised (Santa Cruz) are added to block buffer, 10-15 minutes before use.

Using the Brk antibody we have been able to detect increased Brk expression in eight out eleven paraffin embedded archival breast tumors examined thus far. In two tumors, Brk expression was significantly higher than in other samples. We are repeating these experiments to determine if these tumors have distinct characteristics. Since we are using archival tissues from a tissue bank, it is possible that some of the differences in Brk tumor expression that we have detected thus far are related to differences in the fixation and processing of the tissue.

Brk was first cloned from metastatic breast tumor mRNA, and its expression was not detected in any normal cell type examined. Since we cloned the mouse homologue Sik from mRNA isolated from a healthy mouse intestine, we decided to determine if Brk is expressed in the normal human gastrointestinal tract. To examine Brk expression in the human gastrointestinal tract, biopsy samples from esophagus, stomach, duodenum, and colon epithelia were obtained and total proteins were extracted. The pinch biopsy samples were composed primarily of surface epithelial tissue, enriched in epithelial cells that would possible express Brk. Using the anti-Brk polyclonal antibody, we detected Brk protein in all of the normal human gastrointestinal tissues that we examined.

To determine the pattern of expression of *brk* mRNA expression in the human colon, we performed a series of in situ hybridization experiments using paraffin embedded tissues. We found *brk* gene expression is induced in the upper half of the crypt compartment, where cells have ceased proliferating and have started the process of terminal differentiation, and in differentiated surface epithelial cells. These data coincide with the findings obtained for the murine homologue *sik*, which is expressed in differentiating epithelial cells immediately above zones of proliferation (9). Brk protein was detected by indirect immunofluorescence using the Brk polyclonal antibody, and the protein expression pattern closely parallels the mRNA expression pattern. Expression of Brk in normal human intestinal tissue appears significantly higher that in normal breast tissue.

Conclusions

During our first year of funding we have generated a number of necessary reagents for studying the function of the breast tumor kinase Brk and its mouse homologue Sik. These include wildtype and mutant Sik expression constructs which will allow us to begin to manipulate of expression of Sik in cell lines and mice. We have also made expression constructs for the production of a variety of different Sik-GST fusion proteins, which will be valuable for identifying Sik protein interactions.

We have characterized antibodies that will react with Sik and Brk proteins and identified antibodies that will work well for western blotting, immunoprecipitations, and immunohistochemistry. With these reagents we have begun our analyses of Brk expression during mouse development and in human tissues. We have found that Sik is difficult to detect during normal mouse breast development. Thus far we have only detected significant RNA expression in lactating mammary gland tissue. This is in contrast to the much higher levels of expression detected in the differentiating cells of the skin and intestine. We have detected significant levels of Brk expression throughout the human gastrointestinal cells that are undergoing terminal differentiation. This expression pattern indicates a potential role for Brk during the differentiation of the rapidly regenerating linings of the gastrointestinal tract. We are now preparing a manuscript describing these data (22).

In normal human archival breast tissue we have not detected significant levels of Brk. In contrast, Brk expression was detected in 8 out of 11 breast tumors examined. In two of these breast tumors, Brk was expressed at a significantly higher level and we are currently investigating the significance of this finding. The data support a potential role for Brk in the development of breast tumors.

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